ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, May 2007, p. 1741–1752 Vol. 51, No. 5
0066-4804/07/$08.00+0 doi:10.1128/AAC.01459-06

Roxithromycin Favorably Modifies the Initial Phase of Resistance against Infection with Macrolide-Resistant *Streptococcus pneumoniae* in a Murine Pneumonia Model[▽]

Yasuki Yasuda,[1] Kei Kasahara,[2] Fumiko Mizuno,[1] Kazuyuki Nishi,[1] Keiichi Mikasa,[2] and Eiji Kita[1]*

Department of Bacteriology[1] and Center for Infectious Diseases,[2] Nara Medical University School of Medicine, Kashihara, Nara 634-8521, Japan

Received 20 November 2006/Returned for modification 22 December 2006/Accepted 5 March 2007

Sub-MIC levels of macrolides down-regulate bacterial virulence factors and suppress inflammatory processes. The ability of macrolides to reduce the production of pneumolysin has been shown to explain the discrepancy between in vitro resistance and outcomes with macrolides against macrolide-resistant *Streptococcus pneumoniae*. In this study, we determined whether the ability of macrolides to regulate inflammatory processes is beneficial for innate resistance to macrolide-resistant pneumococci in a murine pneumonia model. Among the macrolides tested, only roxithromycin did not affect in vitro pneumococcal virulence factors at sub-MIC levels. Roxithromycin (1.25 to 10 mg/kg of body weight/day) was administered to mice by oral gavage for 3 days before infection with a resistant strain of *S. pneumoniae*. We evaluated the efficacy of the treatment by determining mouse survival curves and by measuring bacterial burdens and several inflammatory parameters in the airways. Pneumolysin and PspA in infected lungs were examined by Western blot assay. Roxithromycin at doses of ≥5 mg/kg/day increased the median survival time and retarded bacteremia without suppressing the production of pneumolysin and PspA in infected lungs. This treatment reduced matrix metalloproteinase-7 expression and activation and keratinocyte-derived chemokine production in the lungs, while it increased mononuclear cell responses in the lungs, with enhanced bacterial clearance. Concentrations of roxithromycin in plasma and tissues were below the MICs for the inoculated strain during infection. The treatment also reduced inflammatory responses to killed pneumococci in the lungs. These results suggest that the modification by roxithromycin of airway inflammatory responses, including those of matrix metalloproteinase-7 and phagocytes, is beneficial for initial resistance to macrolide-resistant pneumococci.

Streptococcus pneumoniae is the most prevalent pathogen associated with community-acquired pneumonia, and the emergence of antibiotic resistance among pneumococcal isolates linked to community-acquired pneumonia has been of great concern. Especially, the incidence of macrolide-resistant *S. pneumoniae* is rapidly increasing (11, 22). Nevertheless, macrolide compounds have been empirically among the first-line drugs for the treatment of outpatients with community-acquired pneumonia. Such empirical therapy is likely to involve the risk of exacerbating the disease in cases of infection with macrolide-resistant *S. pneumoniae* (31, 32). However, despite the appreciable level of macrolide resistance, concordance between in vitro macrolide susceptibility and clinical outcomes among patients with community-acquired pneumonia is lacking (5, 34); the exact clinical relevance of in vitro findings has yet to be determined. A recent report (12) has shown that the ability of macrolides to reduce pneumococcal virulence factors may account for the discordance between the in vitro resistance of pneumococci and the conservative clinical effects of macrolides. In addition to the suppressive effects of macrolides on microbial virulence factors (12, 27, 35, 42, 45), however, many macrolide compounds, especially those belonging to the 14-membered-ring group, exhibit a variety of regulatory effects on inflammatory processes (reviewed in references 8 and 29). Therefore, it is important to determine whether macrolides can enhance host resistance against macrolide-resistant *S. pneumoniae* only by their ability to regulate inflammatory processes.

Microbial infection in the airway rapidly induces the shedding of ectodomains of syndecan-1, the most abundant heparan sulfate proteoglycan in normal respiratory epithelia (41). We previously observed that a virulent strain of *S. pneumoniae* in mice provokes greater shedding of syndecan-1 ectodomains in the airways than an attenuated strain (25). Syndecan-1 shedding is mediated by host proteinases; the most active enzyme is a member of the matrix metalloproteinase (MMP) family. Among MMPs, MMP-7 (matrilysin) directly stimulates the shedding of syndecan-1 ectodomains from lung epithelia (30). Moreover, MMP-7 is rapidly and strongly expressed in human and murine mucosal epithelial cells in response to brief bacterial exposure; this expression is involved in the initiation of inflammatory processes (9, 33). Thus, it is assumed that virulent pneumococci rapidly induce the expression and activation of MMP-7, thereby causing the shedding of a large amount of syndecan-1 ectodomains in the airway.

Among 14-membered-ring macrolides, erythromycin (ERY) (15, 19) and clarithromycin (CLR) (7, 16) have strong effects on the regulation of inflammatory responses and microbial virulence factors. In addition, azithromycin (AZM), one of the 15-membered-ring macrolides, has been shown to have similar

* Corresponding author. Mailing address: Department of Bacteriology, Nara Medical University School of Medicine, Kashihara, Nara 634-8521, Japan. Phone: 81-744-29-8839. Fax: 81-744-29-7375. E-mail: eijikita@naramed-u.ac.jp.

▽ Published ahead of print on 12 March 2007.

activity (12). In contrast, sub-MIC levels of roxithromycin (RXM) are reported to be less effective in reducing bacterial virulence factors (13), despite the fact that this macrolide exhibits a variety of immunomodulatory activities (28, 43). Furthermore, it deserves attention that 14-membered-ring macrolides, including RXM, have the capability of suppressing several types of MMPs in eukaryotic cells (18, 21). We therefore sought to address whether sub-MIC levels of 14-membered-ring macrolides directly regulate the innate response to macrolide-resistant *S. pneumoniae* by down-regulating MMP-7. For this purpose, we first examined the abilities of commonly used macrolide antibiotics (ERY, RXM, CLR, and AZM) at sublethal concentrations to affect pneumococcal virulence factors; we showed that sub-MIC levels of RXM did not suppress the production of pneumococcal virulence factors in vitro. Thereafter, we examined the effects of sub-MIC levels of RXM on local responses to macrolide-resistant *S. pneumoniae* in a murine pneumonia model.

In the present study, we have provided the first evidence showing that sub-MIC levels of RXM enhance local innate resistance to macrolide-resistant *S. pneumoniae*, independently of the direct action of RXM against the pathogen. Furthermore, this enhancement seems to be associated with the ability of RXM to down-regulate MMP-7 in addition to its regulatory effects on neutrophils and macrophage functions in the airway.

MATERIALS AND METHODS

Mice. Three- to 4-week-old, male CBA/J mice (specific-pathogen free) were obtained from Charles River Japan (Kanagawa). The mice were housed four per cage and were allowed food and water ad libitum. The mice were allowed to acclimatize for 7 to 12 days prior to the initiation of experimental procedures and were used for experiments when they weighed 28 to 30 g. The experimental protocols were approved by the Ethics Review Committee for Animal Experimentation of the Nara Medical University School of Medicine, Kashihara, Nara, Japan.

Macrolides. RXM (Aventis Pharmaceuticals, Tokyo, Japan), ERY and CLR (Abbott Japan, Osaka), and AZM (Pfizer Japan Inc., Tokyo) were kindly provided by the indicated companies. RXM, ERY, and CLR were dissolved in 95% ethanol at 200 mg/ml (stock solutions). AZM was suspended to a final concentration of 30 mg/ml with 0.1% carboxymethyl cellulose (stock solution). These stock solutions were kept at −20°C and were further diluted with sterile saline to yield the appropriate drug concentrations in the final administration volume of 100 μl. Diluted drug solutions were stored at 4°C for no more than 24 h.

Antibiotics were administered via oral gavage. ERY at 10 or 20 mg/kg of body weight/day and CLR at 5 or 10 mg/kg/day were administered twice daily at 12-h intervals. AZM was administered at 5 or 10 mg/kg/day once daily. RXM was administered twice daily at a dose of 1.25, 2.5, 5.0, 7.5, or 10 mg/kg/day. The control regimen was 100 μl of saline. The treatment dose (defined as a high dose) of each macrolide for a 30-g mouse was determined, based on the weight ratio, from a therapeutic dose for a 60-kg adult (therapeutic doses of ERY, AZM, CLR, and RXM for a 60-kg adult patient are 1,200, 500, 400, and 300 mg per day, respectively). Half of the amount of the high dose was used for the low-dose treatment. Treatment was initiated 3 days before infection and continued for 3 days. For the in vitro study, 0.005% ethanol in phosphate-buffered saline (PBS; 10 mM, pH 7.2) was used as a vehicle solution for RXM, CLR, and ERY and 0.005% carboxymethyl cellulose in PBS was used for AZM.

***S. pneumoniae*.** Three strains of *S. pneumoniae* were used in this study. Strain NMU112 is a clinical isolate harboring *erm*(B) and *mef*(A) genes, as confirmed by PCR at the Nara Medical University School of Medicine hospital; the MICs of ERY, RXM, CLR, and AZM were ≥256 μg/ml. The intratracheal 50% lethal dose for CBA/J mice was approximately 500 CFU. The other strains, NMUP45 (serotype 4; MICs of ERY, RXM, and CLR, 2 μg/ml; MIC of RXM, 4 μg/ml) and NMU605 (serotype 3; MICs of ERY and CLR, 0.03 μg/ml; MIC of RXM, 0.06 μg/ml; MIC of AZM, 0.125 μg/ml), were also clinical isolates. The MICs of each macrolide were measured according to broth microdilution techniques using Mueller-Hinton broth with 3% lysed horse blood (Becton Dickinson Microbiology Systems, Cockeysville, MD) as described by the NCCLS (36). The determination of capsular serotypes was performed by the slide agglutination method using a panel of commercially obtained antisera ("Seiken" set *S. pneumoniae* antisera; Seiken Co., Tokyo, Japan) as previously described (23). The antisera included polyclonal antibodies for 39 frequently isolated serotypes and/or groups of *S. pneumoniae*.

The organisms were grown in Todd-Hewitt broth (Difco Laboratories, Detroit, MI) supplemented with 0.5% yeast extract (THY) to mid-log phase at 37°C; the cells were harvested and suspended to a density of 5×10^7 CFU/ml in THY plus 10% glycerol and frozen in aliquots at −20°C until use. Before use, one of the frozen stocks was thawed and cultured on THY agar containing 5% horse blood at 37°C for 18 h. Single colonies were expanded in THY by incubation at 37°C for 6 to 8 h to mid-log phase (A_{600}, 0.2 to 0.4), harvested by centrifugation, and resuspended in PBS containing 5% (vol/vol) heat-treated fetal bovine serum (PBS-FBS; GIBCO, Invitrogen Corp., Carlsbad, CA) for intratracheal infection or in cell culture medium for the in vitro infection. The actual number of CFU administered was determined by plating serial dilutions of a starting inoculum on THY agar supplemented with 200 μg of ERY/ml (ERY plate). Throughout this study, ERY plates were used only for strain NMU112 while THY agar without ERY was used for strains NMUP45 and NMU605.

Determination of RXM concentrations. The concentrations of RXM in plasma and lung tissues were measured by high-performance liquid chromatography (HPLC) with electrochemical detection according to the method of Patel et al. (39), except that the mobile phase consisting of acetonitrile, methanol, and 0.5% ammonium acetate (39:11:50 [vol/vol/vol]; pH 5.5) was delivered at a flow rate of 1 ml/min. The internal standard was erythromycin. For this study, blood samples were spun at 1,000 × *g* for 10 min and plasma was frozen until the assay. Lungs were frozen, homogenized in 2 ml of PBS with a tissue homogenizer (Nichionrika, Tokyo, Japan), and then sonicated for 2 min before the assay. To 200 μl of each sample, 50 μl of the internal standard was added, and then each sample was alkalinized with 200 μl of 0.1 M sodium carbonate. The diluted sample was extracted with *tert*-methyl-butyl ether (Wako Pure Chemicals, Osaka, Japan) and separated by centrifugation, and the ether layer was evaporated to dryness. The residue was reconstituted in 200 μl of the mobile phase, and the aqueous layer (80 μl) was injected into the HPLC system. The standard curve was constructed by weighted linear regression of the peak area versus concentration and was linear, as follows: 0.15 to 10.5 μg/ml in plasma and 0.4 to 11.8 μg/ml in lung homogenates. The lowest concentration of the prepared standards was defined as the limit of detection for each assay. A plasma sample and lung homogenate from the same mouse were prepared, and five mice at each time point were used.

Infection with *S. pneumoniae*. *S. pneumoniae* NMU112 was grown to mid-log phase in THY as described above, and a 20-μl volume of the bacterial suspension (2.5×10^6 CFU/ml) in PBS-FBS was inoculated into the oropharynges of mice slightly anesthetized intraperitoneally with ketamine (6 mg/kg of body weight) plus xylazine (1 mg/kg). Infection was performed 24 h after the last dose. In one experiment, UV ray-killed NMU112 pneumococci were intratracheally inoculated into mice. A suspension of NMU112 pneumococci (5×10^9 CFU/ml) in a petri dish was exposed to a 254-nm UV lamp at a distance of 15 cm (930 μW/cm^2) for 20 min. Sterilization was verified by the plating of 100 μl of the suspension onto THY agar plates. The killed bacteria were centrifuged and suspended in PBS-FBS, and 20 μl of the suspension was inoculated intratracheally into mice.

Collection of BALF, blood, and lungs. Mice were killed with an overdose of CO_2. The trachea of each mouse was cannulated with a sterile 22-gauge catheter, and the right hilum was clamped. Bronchoalveolar lavage was performed by the instillation of three 0.8-ml aliquots of sterile ice-cold PBS-FBS into the left lung. The bronchoalveolar lavage fluid (BALF) retrieved from all the mice in each treatment group was combined (~2.2 ml). The total cell counts were determined using a Z2 Coulter particle count and size analyzer (Beckman Coulter, Fullerton, CA), and differential cell counts were performed with cytospin preparations stained with Diff-Quik (Baxter, Deerfield, IL). Supernatants of the combined BALF were serially diluted with sterile PBS-FBS and cultured on ERY plates. After lavage, blood was extracted from the heart, and the left lung was excised and homogenized at 4°C in 2 ml of PBS-FBS by using a sterile tissue homogenizer. Appropriate dilutions of blood samples and lung homogenates in PBS-FBS (100 μl) were plated onto ERY plates and incubated at 37°C for approximately 24 h for CFU determinations. The minimum number of CFU detectable in the lungs was $\log_{10}$ 1.83. For the cell analysis, a total of six mice per group at each time point were used in two separate experiments. For bacterial counts, three mice per group at each time point were used in three separate experiments.

Measurement of syndecan-1 ectodomains by ELISA. We constructed an enzyme-linked immunosorbent assay (ELISA) for mouse syndecan-1 ectodomains. The system used a solid-phase monoclonal 281-2 antibody (1.25 μg/ml; rat

immunoglobulin G2aκ [IgG2aκ[; Research Diagnostics Inc.) in the wells of 96-well plates (BD Biosciences), and the coated plates were blocked with 5% nonfat dry milk (Sigma-Aldrich) in PBS containing Tween 20 (0.1% [vol/vol]). A biotinylated monoclonal 281-2 antibody (1.10 μg/ml; BD Biosciences) was used as a second antibody. The detection steps included the use of streptavidin-horseradish peroxidase (HRP) and tetramethylbenzidine as chromogens. For each analysis, 100 μl of BALF was used, and samples from individual mice (*n*, 3 per group at each time point) were assayed in triplicate. The calibrations on each microtiter plate included syndecan-1 ectodomains purified from NMuMG 29 cells (a mouse mammary cell line) (3) as a standard. Optical densities were determined using a microtiter plate reader (model 3550; Bio-Rad Laboratories) at 450 nm. The performance of the present ELISA system was as follows. The range of detection was 2.5 to 245 ng/ml (sensitivity, <2.5 ng/ml), and no cross-reacting molecules in BALF were found. The range of concentrations in normal BALF (from 16 normal mice) was 8.5 ± 2.3 ng/ml; a correlation coefficient (linear regression of concentrations in BALF samples versus the expected concentration) was 0.97. The intra- and interassay coefficients of variation were 4.8% (range, 4.5 to 6.2%) and 6.2% (range, 5.8 to 7.3%), respectively.

ELISA for mouse chemokines. Keratinocyte-derived chemokine (KC) and monocyte chemoattractant protein-1 (MCP-1) levels in BALF and lung homogenates were determined by sandwich ELISA (Quantikine; R & D Systems, Minneapolis, MN). A homogenate of the left lung (*n*, 3 per group at each time point) was diluted 1:1 in lysis buffer containing 2× protease inhibitor cocktail (Roche Diagnostics, Basel, Switzerland). After 30 min of incubation on ice, the mixture was centrifuged at 12,000 × *g* for 20 min at 4°C and supernatants were stored at −80°C until the assay. Total protein was measured using bicinchoninic acid protein assays (Bio-Rad). Both ELISA kits detected as little as 2 pg/ml of the respective recombinant proteins.

Reverse transcriptase PCR (RT-PCR). The left lung was frozen and crushed using a sterile, nitrogen-cooled homogenizer. Total RNA was extracted using the TRIzol reagent (Invitrogen), and 1 μg of total RNA was transcribed into cDNA by using a preamplification system (Invitrogen) and Moloney murine leukemia virus reverse transcriptase (Invitrogen). Quantitative real-time PCR analysis was performed by using an ABI Prism 7700 sequence detector system (PE Applied Biosystems, Foster City, CA). Primer-probe sets for chemokines and cytokines were purchased from PE Applied Biosystems. PCR was performed with the TaqMan universal PCR master mix (PE Applied Biosystems) by using 1 μl of cDNA in a 20-μl final reaction mixture volume. The PCR thermal cycle conditions were as follows: an initial step at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The levels of each mRNA were divided by the levels of mRNA for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and mRNA levels in control mice were assigned an arbitrary value of 1. In these assays, three mice per group at each time point were used.

Southern hybridization assay. Total RNA (500 ng) was mixed with mouse GAPDH-specific or MMP-7-specific primers (17); one-step RT-PCR was performed using SuperScript III one-step RT-PCR with Platinum *Taq* DNA polymerase according to the instructions of the manufacturer (Invitrogen). The sequence of the sense primer for MMP-7 was 5′-TGGAGTGCCAGATGTTG CAG-3′, and the antisense sequence was 5′-TTTCCATATAGCTTCTGAATG CCT-3′ (47). The MMP primers were designed to detect the mouse MMP-7 gene and not to hybridize with genes for other known MMPs. The PCR products were separated on a 2% agarose gel, blotted onto Hybond-N^+ membranes (Amersham), and hybridized overnight at 42°C to alkaline phosphatase-labeled 3′ MMP-7 cDNA probes (47) or cDNA probes specific to the mouse GAPDH gene (10). Both probes were made using direct labeling and detection systems (Amersham). The chemiluminescence of CDP-Star hybridized blots was detected with CDP-Star reagent (Amersham), and blots were exposed to Hyperfilm ECL for 2 h (Amersham). RNA samples were extracted from three mice per group at each time point, and individual samples were assayed in two separate experiments.

Western blot for PLY and PspA. The presence of pneumolysin (PLY) and pneumococcal surface protein A (PspA) in lung tissue was determined by Western blot analysis. The left lungs of mice (*n*, 3 per group at each time point) were homogenized in 50 mM Tris-HCl buffer (pH 7.5) containing 75 mM NaCl and 1% Triton X, and the homogenate was centrifuged at 12,000 × *g* for 20 min at 4°C. The supernatant (lung extract) was used for Western blot analysis. One milliliter of lung extract (1 mg of protein) was incubated for 1 h at 4°C with 85 μg of monoclonal murine antibodies (IgG) to recombinant PLY (NCL-SPNm; Novocastra Laboratories, Newcastle, United Kingdom) or 5 μg of goat anti-PspA antibodies (IgG; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), both of which were coupled to protein G beads (Amersham). The beads were washed three times with 2 ml of diluted lysis buffer before boiling in sodium dodecyl sulfate (SDS) sample buffer. After SDS–12% polyacrylamide gel electrophoresis (SDS–12% PAGE), proteins were transferred onto nitrocellulose by using a semidry transfer apparatus (ATTO Cooperation, Tokyo, Japan). To prevent nonspecific reactions, both antibodies were incubated at 4°C for 12 h with acetone powder from normal lung homogenates before use; with this procedure, immunoreactions were not observed by Western blotting using lung extracts and these antibodies. Membranes were probed with either HRP-conjugated goat anti-mouse IgG (1:2,000; Amersham) or HRP-conjugated donkey anti-goat IgG (1:2,500; Santa Cruz Biotechnology). The immunoblots were developed with metal-enhanced DAB (Pierce Biotechnology, Rockford, IL). To determine relative amounts of PLY and PspA, the reaction mixture was exposed to Hyperfilm ECL (Amersham); densitometries of the films were determined with NIH Image software (version 1.62). The values were expressed as arbitrary optical densitometric units, and results (n = 5 to 6) were presented as percentages of units corresponding to control mice.

A Western blot assay for PLY and PspA produced by cultured pneumococci was performed as follows: strain NMU112 (5×10^7 CFU/ml) in THY was incubated in the presence of RXM (0.5 to 5 μg/ml) at 37°C for 12 h. After incubation, bacteria were harvested, washed, and lysed with lysis buffer containing 2× protease inhibitor cocktail by using a sonicator. Whole-cell lysates of strain NMU112 were subjected to SDS-PAGE. The Western blot procedure was performed as described above. In this assay, the lysate was prepared from two cultures per dose in each experiment, and three separate experiments were performed (n = 6). This assay included ERY (10 μg/ml), CLR (5 μg/ml), and AZM (5 μg/ml) (*n*, 2 per group in each experiment).

Zymography and Western blotting for MMP-7. Casein zymography was performed with lung extract (100 μg of protein) according to the method previously described (14) by using NOVEX 4 to 16% zymogram blue casein gels (Invitrogen, Carlsbad, CA). To determine relative levels of activity of mature MMP-7, the zymograms were inverted following staining and destaining; densitometry levels were measured by digital image analysis (NIH Image software). The values were expressed as arbitrary optical densitometric units, and results were presented as percentages of units corresponding to the extracts from control mice. This assay was repeated three times using five or six mice for both groups (the RXM-treated and control groups) at each time point.

In order to determine the molecular sizes of caseinolytic enzymes detected by zymography, the lung extract (100 μg) was subjected to SDS-PAGE and a Western blot assay using rabbit anti-human MMP-7 polyclonal antiserum (AnaSpec, Inc., San Jose, CA) and HRP-conjugated donkey anti-rabbit IgG (Amersham) (14). The antibody to MMP-7 recognizes the C terminus of human MMP-7, the sequence of which is identical that of mouse MMP-7, and detects both the precursor (28-kDa) and mature (19-kDa) forms of mouse MMP-7. The immunoblots were developed with metal-enhanced DAB (Pierce Biotechnology) as described above. When the extract of infected mouse lungs was pretreated with excess amounts of this antibody, the levels of caseinolytic activity of both the precursor and mature forms in the extract were reduced by more than 95% compared with those in the untreated extract.

MPO activity. Myeloperoxidase (MPO) activity in lung tissue was measured using an MPO assay kit (Cytostore, Alberta, Canada). After blood extraction, the left lungs of mice (*n*, 4 per group at each time point) were removed and weighed and immediately frozen. Homogenization of the frozen lungs and the assay were carried out according to the manufacturer's instructions. MPO activity was expressed as units per milligram of protein in lung tissue.

Bactericidal assay. Blood and lungs were obtained under aseptic conditions at 24 h after the last dose (at the time of infection). Serum aseptically prepared from the blood was heated at 56°C for 30 min. The lungs were homogenized at 4°C in 1 ml of PBS-FBS by using a sterile tissue homogenizer (Nichionrika) and centrifuged at 12,000 × *g* for 20 min at 4°C; supernatants were membrane-filtered before use. Both samples were dispensed in 90-μl aliquots into three sterile plastic tubes containing 4×10^3 CFU of pneumococci in 10 μl of PBS-FBS. The sealed tubes were rotated end-over-end for 1 h at 37°C, and the mixture (50 μl) was plated onto THY agar.

Histopathological study. Lungs for histological examination (*n*, 4 per group) were harvested 30 h after infection, fixed in 10% formalin, and embedded in paraffin. Five-micrometer sections were stained with hematoxylin and eosin.

Statistics. Data are expressed as means ± standard errors of the means (SEM), except the data on RXM concentrations in plasma and lung tissue. Differences in cell counts, quantities of chemokine and syndecan-1, numbers of viable bacteria, and MPO activity levels were statistically evaluated using analysis of variance. The Mann-Whitney U test with the Bonferroni correction was used to compare percentages of surviving pneumococci in the bactericidal assay, percentages of infiltrating cell populations in BALF, and results from the densitometric analysis of Western blotting and real-time PCR. Survival analysis was

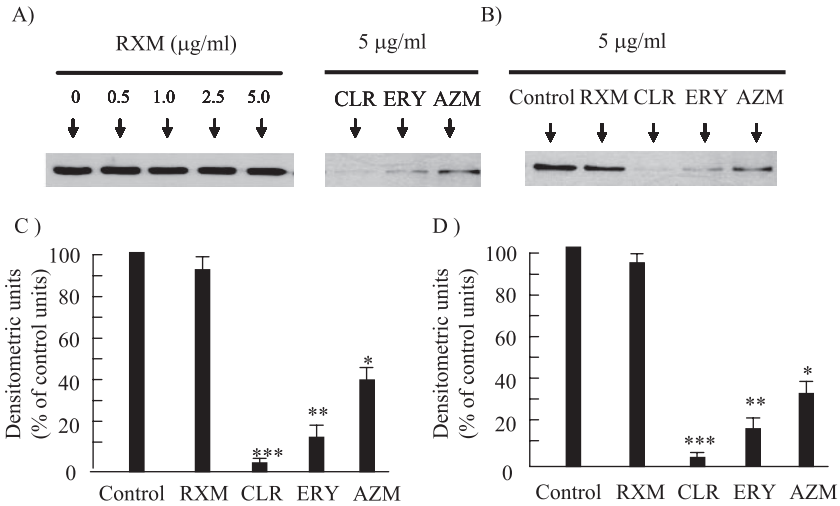

FIG. 1. Effect of macrolides on the production of PLY and PspA by MRSP in vitro. A Western blot assay for PLY (A) and PspA (B) was performed with whole lysates of strain NMU112 (5×10^7 CFU/ml) harvested after 12 h of culture in the presence of sub-MIC levels of macrolides: 0.5 to 5 μg/ml of RXM or 5 μg/ml of ERY, CLR, and AZM. Results representative of those from two separate experiments are shown. The signal intensities of bands for PLY (C) and PspA (D) were determined using a computerized densitometer; in this assay, whole lysates from the culture were prepared in the presence of RXM (5 μg/ml), CLR (5 μg/ml), AZM (5 μg/ml), or ERY (10 μg/ml). Data are representative of results from three separate experiments and are expressed as mean percentages of the intensity of bands for PLY and PspA in the lysate of the pneumococci cultured without macrolides (control). The error bars represent SEM (n, 5 to 6 at each time point). *, P of <0.05; **, P of <0.01; ***, P of <0.0075 for comparison with controls.

performed using the log rank test, and survival rates were calculated via the Kaplan-Meier method. Statistical significance was set at a P value of <0.05.

RESULTS

Effects of macrolide compounds on the production of PLY and PspA. First, we examined the abilities of four macrolide compounds (CLR, ERY, AZM, and RXM) to affect the production of pneumococcal virulence factors (PLY and PspA) by Western blot assay. A Western blot assay for these proteins was performed with the lysate of strain NMU112 (5×10^7 CFU/ml). Representative results from Western blot analysis are shown in Fig. 1A and B. Culture with 5.0 μg/ml of RXM did not suppress PLY production, while the same concentration of CLR ($P < 0.0075$) and AZM ($P < 0.05$) and 10 μg/ml of ERY ($P < 0.01$) reduced it significantly (Fig. 1C). CLR (5.0 μg/ml), ERY (10 μg/ml), and AZM (5.0 μg/ml) significantly inhibited the production of PspA compared with that in the control, but 5 μg/ml of RXM did not (Fig. 1D). Although the concentrations used for this assay were higher than clinically achievable concentrations, the published human pharmacokinetic data reveal that macrolide concentrations in pulmonary epithelial lining fluid and alveolar macrophages are routinely higher than those in plasma (40). Since macrolide-resistant *S. pneumoniae* first encounters pulmonary epithelial lining fluid and/or phagocytes in the airway, we chose these drug concentrations. In these analyses, there was no difference in the numbers of CFU recovered after 12 h of incubation in the presence and absence of these macrolides.

Effect of macrolides on mouse survival. Next, we determined whether CLR, ERY, and AZM can increase the survival rates of mice after infection with the highly resistant strain NMU112. The survival rates of mice treated with ERY (10 or 20 mg/kg/day), CLR (5 or 10 mg/kg/day), and AZM (5 or 10 mg/kg/day) before infection with NMU112 are shown in Fig. 2. Treatment with these macrolides, at either the low or high dose, appeared to improve the survival rates compared with those of controls. The median survival time (MST) of the control mice was 2.65 days. The MSTs corresponding to the low doses of the macrolides were as follows: 6.82 days for CLR, 5.84 days for AZM, and 5.27 days for ERY. The high doses of these macrolides slightly, but not significantly, increased each MST by 1.49 to 1.63 days over that associated with the low dose of each drug; the MSTs for high-dose-treated mice were 7.33 days for AZM, 6.78 days for ERY, and 8.45 days for CLR.

Next, we performed the same study with RXM, since this macrolide did not suppress in vitro the production of microbial virulence factors. All mice receiving either saline (control) or 1.25 mg/kg/day of RXM died within 3 days after infection (Fig. 3A); severe hemorrhagic pneumonia developed along with pneumococcal bacteremia in both groups of infected mice. In contrast, treatment with RXM at doses of ≥2.5 mg/kg/day resulted in longer MSTs; 5 mg/kg/day of RXM corresponded to a significantly longer MST (5.8 days) than the control treatment (2.65 days; $P < 0.05$). However, neither 7.5 mg/kg/day (MST, 6.3 days) nor 10 mg/kg/day (MST, 6.6 days) of RXM generated a significant increase in the MST compared to that corresponding to the 5-mg/kg/day dose of the drug. Histologically, acute pneumonic change diminished between days 3 and 4 of infection in RXM-treated mice (≥5 mg/kg/day), though all of the treated animals died within 8 days. Our unpublished observation showed that treatment with RXM at ≥2.5 mg/kg/day against NMU605 and that at ≥5 mg/kg/day against NMUP45 resulted in no death of infected mice, possibly due to the direct antimicrobial activity of RXM.

Lung extract obtained at 24 h after the last treatment with 10 mg/kg/day of RXM did not kill NMU112 pneumococci after

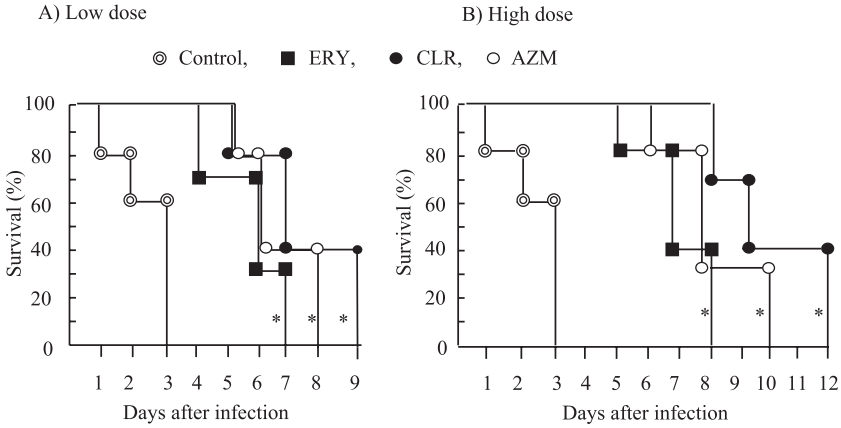

FIG. 2. Survival curves for strain NMU112-infected mice treated with ERY, CLR, or AZM. Kaplan-Meier survival curves for infected CBA/J mice receiving low doses of ERY (10 mg/kg/day, administered twice daily), CLR (5 mg/kg/day, administered twice daily), and AZM (5 mg/kg/day, administered once daily) (A) and high doses of ERY (20 mg/kg/day, administered twice daily), CLR (10 mg/kg/day, administered twice daily), and AZM (10 mg/kg/day, administered once daily) (B) for 3 days before infection. Infection with 5×10^4 CFU of strain NMU112 was performed 24 h after the last dose. Data were obtained from three separate experiments (10 mice per group in each experiment). *, P of <0.05 for comparison with the control group.

1 h of incubation, while killing rates for strains NMU605 and NMUP45 were significantly higher ($P < 0.025$) upon the exposure of these strains to extracts from mice treated with RXM at ≥2.5 and ≥5 mg/kg/day, respectively (Fig. 3B). In addition, heat-treated serum samples from mice receiving 10 mg/kg/day of RXM did not reduce numbers of CFU of strain NMU112 after 1 h of incubation (mean reduction, 2.2% ± 1.3% of the numbers of CFU in the inoculum).

Next, we measured RXM concentrations in plasma and lung tissue by HPLC; mice weighing 30 g were treated for 3 days with 5 mg/kg/day of RXM (i.e., 0.075 mg given twice daily for six doses). The RXM concentrations in plasma and lung tissue 24, 48, and 72 h after the last dose are shown in Table 1. At the tested drug concentrations, which were below the MIC for strain NMU112, the synthesis of pneumococcal virulence factors might not be affected in plasma and lung tissue after infection. Thus, treatment with RXM (5 mg/kg/day) was expected to modify the host response to macrolide-resistant *S. pneumoniae* in the lungs without reducing pneumococcal virulence.

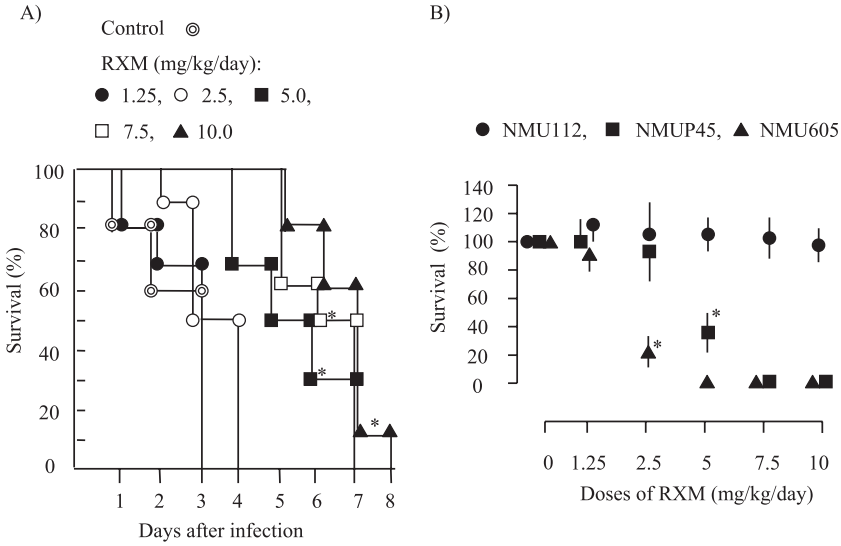

FIG. 3. Survival curves for strain NMU112-infected mice treated with RXM and resistance of the bacterial strain against lung extract. (A) Kaplan-Meier survival curves for infected CBA/J mice receiving various doses of RXM for 3 days before infection. Infection was performed 24 h after the last dose. Data were obtained from two separate experiments (10 mice per group in each experiment). *, P of <0.05 for comparison with the control group. (B) Resistance of strain NMU112 to the lung extract prepared 24 h after the last dose of RXM. The reaction mixture consisted of 90 μl of extract and 10 μl of bacterial suspension (4×10^3 CFU) and was incubated for 1 h; for each dose, samples from three mice were obtained, and the assay was performed in triplicate for individual samples. Data were obtained from three separate experiments. Data are represented as the mean percentages of bacteria in the starting inoculum (4×10^3 CFU) surviving in the mixture. The error bars represent SEM. *, P <0.025 for comparison with the starting inoculum.

TABLE 1. Concentrations of RXM in plasma and lung tissue[a]

Time (h) after the last dose (day)	Concn (μg/ml) of RXM in:	
	Plasma	Lung tissue
24 (0)	3.87 ± 0.42	5.22 ± 0.36
48 (1)	1.36 ± 0.33	2.87 ± 0.25
72 (2)	0.54 ± 0.13	0.65 ± 0.22

[a] Concentrations of RXM in plasma and lung tissue were measured by HPLC at 24, 48, and 72 h after the administration of the last dose of RXM. A plasma sample and a lung homogenate from the same mouse ($n = 5$) were prepared at each time point. Data are expressed as the means ± standard deviations.

Thus, in the following experiments to assess the direct effect of 14-membered-ring macrolides on the host response, we used the 3-day treatment with RXM at a daily dose of 5 mg/kg prior to infection.

Measurement of numbers of CFU in BALF, lungs, and blood. The numbers of viable pneumococci in the lungs and BALF of RXM- and saline (control)-treated mice after infection with strain NMU112 were determined (Fig. 4). During the first 12 h of infection, numbers of CFU in lungs and BALF of both mouse groups gradually increased. Between 24 and 48 h, the numbers of pneumococci in the lungs and BALF of the treated mice decreased, while the numbers of CFU in control mice still increased (the difference in numbers of CFU at 48 h was approximately $\log_{10}$ 1.78). We also found that numbers of CFU in the lungs (slope, −0.73; $r^2 = 0.64$; $P < 0.05$) and in BALF (slope, −0.67; $r^2 = 0.72$; $P < 0.05$) obtained after 24 h of infection were inversely correlated with survival times for individual mice (data not shown). Pneumococcal bacteremia occurred in the controls as early as 12 h after infection; the mean $\log_{10}$ numbers of CFU per milliliter of blood in surviving mice were 2.18 at 12 h, 3.80 at 24 h, 4.65 at 36 h, and ≥5.65 at 48 h after infection. In contrast, *S. pneumoniae* was not recovered from the blood of RXM-treated mice during the first 3 days. After day 4, however, the mean $\log_{10}$ numbers of CFU per milliliter of blood in surviving RXM-treated mice gradually increased: 2.14 at day 4, 3.68 at day 5, 4.27 at day 6, and ≥5.11 at day 7. The high dose of CLR, ERY, or AZM delayed the development of bacteremia by 12 h (ERY) or 24 h (CLR and AZM) compared with RXM treatment (data not shown). These results therefore indicated that the slow development of pneumococcal bacteremia is closely related to the longer survival times for individual mice treated with macrolides.

Analyses of infiltrating cells, syndecan-1, and chemokines in the airways. The number of total cells in BALF from control mice increased linearly during the first 36 h, and the number in BALF from RXM-treated mice increased linearly during the first 24 h (Fig. 5A). Among these cells, polymorphonuclear leukocytes (PMNs) were predominant in the controls (Fig. 5B) while mononuclear cells were predominant in the treated animals (Fig. 5C). The level of PMN infiltration in lung tissue from the controls was greater than that in lung tissue from the treated mice after 12 h, as assessed by measuring the levels of MPO activity in lung tissue (Fig. 5D). In parallel to such a difference in the cellular response, KC levels in lung tissue from the treated mice after 12 h were significantly lower than those in tissue from the controls (12 h, $P < 0.05$; 24 and 36 h, $P < 0.01$) (Fig. 6A, left panel). MCP-1 levels in the lungs of treated mice were significantly higher than those in the controls between 12 and 36 h after infection (12 and 36 h, P of <0.05; 24 h, P of <0.01 for comparison with controls), while this protein was produced at the same levels in the two groups during the first 8 h (Fig. 6A, right panel). KC levels in BALF from RXM-treated mice were significantly lower after 8 h (8 h, P of <0.05; 12, 24, and 36 h, P of <0.01 for comparison with controls), while MCP-1 in the treated animals was significantly higher after 8 h (8 h, P of <0.05; 12, 24 and 36 h, P of <0.01 for comparison with controls) (data not shown). These findings were comparable to the results of quantitative analyses of the expression levels of mRNAs for KC and MCP-1 (Fig. 6B) at 8 and 24 h after infection, while message levels for tumor necrosis factor alpha and interleukin-1β did not differ between the two groups. Higher KC levels in BALF were associated with enhanced syndecan-1 shedding in control mice, which lasted until death (Fig. 6C). In the treated mice, the shedding was transient and the levels were significantly lower than those in controls (at 1 and 2 h, $P < 0.05$; after 4 h, $P < 0.01$).

Expression of MMP-7 in infected lungs. Infection induced MMP-7 expression in the lungs of all infected mice as early as 30 min after infection (data not shown), but thereafter, MMP-7 expression levels in control mice became apparently greater than those in the treated animals at each time point (Fig. 7A). By zymography using lung extract obtained at 12 h postinfection, the caseinolytic activity was found to be present in lung extracts from all mice (Fig. 7B). Among the proteolytic bands, a precursor (28 kDa) and a mature form (19 kDa) were identified by Western blot analysis (Fig. 7B). The caseinolytic activity of a mature form in the RXM-treated animals was significantly decreased from 6 to 48 h after infection compared to that in the controls (6 and 48 h, $P < 0.05$; 12 and 24 h, $P < 0.025$) (Fig. 7C).

Effect of RXM on the production of PLY and PspA in infected lungs. In the lungs of the treated mice, PLY (Fig. 8A) and PspA (Fig. 8B) produced by NMU112 did not increase after 24 h of infection as demonstrated by Western blot analysis; the numbers of densitometric units of these proteins in the lungs of the treated mice after 48 h of infection were significantly lower than those in the controls (48 h, $P < 0.05$; 60 h, $P < 0.01$) (Fig. 8C). Since RXM (0.5 to 5.0 μg/ml) did not affect the production of pneumococcal

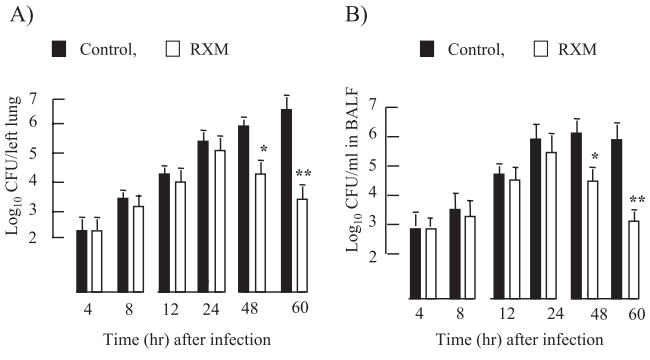

FIG. 4. Enumeration of bacteria in the lungs after infection. Groups of CBA/J mice were intratracheally infected with 5×10^4 CFU of strain NMU112. The bacterial loads in the lungs (A) and BALF (B) were assessed at the indicated time points: three mice per group at each time point were used. Data are representative of results from three separate experiments, and the error bars represent SEM. *, P of <0.01; **, P of <0.005 for comparison with the control group.

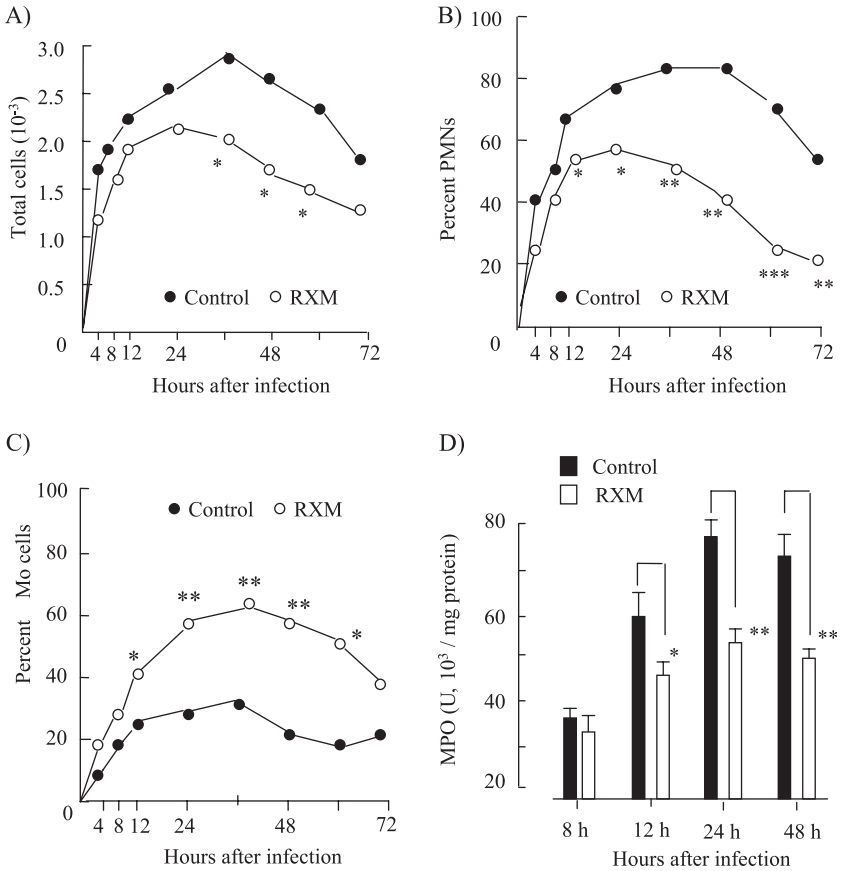

FIG. 5. Analysis of infiltrating cells in the airway after infection. BALF was harvested at the indicated times after infection with 5×10^4 CFU of strain NMU112. (A) Total counts of infiltrating cells in BALF. Percentages of PMNs (B) and mononuclear (Mo) cells (C) among infiltrating cells are shown. Data are representative of results from two separate experiments and are expressed as the mean values ($n = 6$). *, P of <0.05; **, P of <0.025; ***, P of <0.01 for comparison with controls. (D) PMN infiltration into the lungs was estimated by measuring the levels of MPO activity in the lung tissue, shown as the mean values (n, 4 at each time point). The error bars represent SEM. *, $P < 0.05$; **, $P < 0.025$.

virulence factors (Fig. 1), the decrease in these proteins after 48 h of infection appeared to be a result of the decreased bacterial burdens in the lungs (Fig. 4A).

Effect of RXM on host responses to killed pneumococci. Finally, we ascertained whether the reduced PMN-dominant inflammation was really due to the direct effect of RXM on the local response. For this purpose, we inoculated intratracheally 10^8 killed NMU112 organisms into RXM-treated mice. Levels associated with inflammatory responses, including total cell counts (Fig. 9A) and levels of PMN infiltration (Fig. 9B) and MMP-7 expression (Fig. 9C), were significantly reduced in the treated mice compared with those in the controls. Although MCP-1 levels were not measured in this experiment, the levels of expression of mRNA for MCP-1 apparently increased in the lungs of RXM-treated mice by 8 h after inoculation (our unpublished observation). These results indicated that the regulatory effect of RXM on local inflammatory processes in the lungs was due to its direct action on the host response.

DISCUSSION

The present study demonstrates that the treatment of mice with a subtherapeutic dose of RXM for 3 days before infection with macrolide-resistant *S. pneumoniae* improved mouse survival times, which was associated with the delayed occurrence of bacteremia. This effect seems to be due mainly to the regulatory effect of RXM on local host responses, including MMP-7 expression and activation and phagocytic cell functions in the lungs; furthermore, such regulation may occur without the suppression of pneumococcal virulence factors. Our previous study showed that a less virulent strain of *S. pneumoniae* induced lower degrees of syndecan-1 shedding in the airway than a virulent one (25). This difference may be relevant to the distinction in the levels of shedding in the RXM-treated mice and the control mice in this study, since the treated animals were more resistant to strain NMU112 than the controls. Syndecan-1 knockout mice are shown to be resistant to intratracheal infection with *Pseudomonas aeruginosa*; moreover, intratracheal administration of purified syndecan-1 ectodomains into the airways impairs their resistance (38). Considering these findings together, decreased levels of syndecan-1 shedding may reflect the attenuation of infections with these two respiratory pathogens.

MMP-7 directly cleaves syndecan-1 from the surfaces of cells in vitro (30). Thus, the decrease in syndecan-1 shedding observed in RXM-treated mice may have resulted from the suppressed expression or activation of MMP-7. MMP-7 activity as

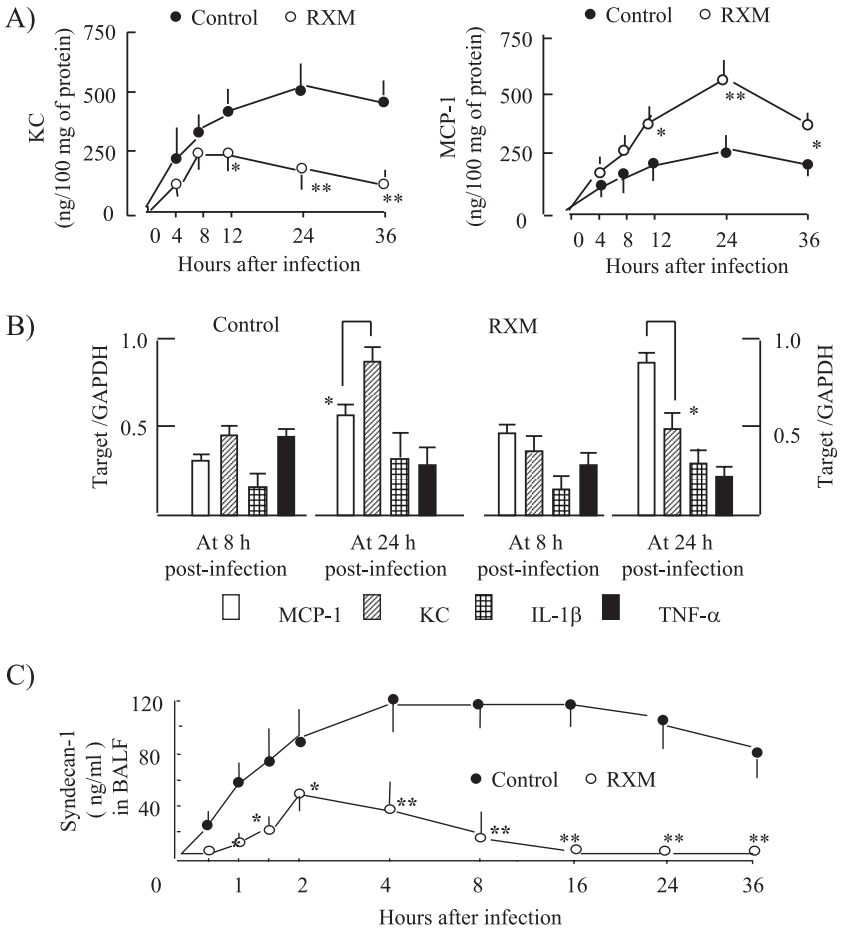

FIG. 6. Measurement of chemokines and syndecan-1 by ELISA and quantitation of mRNAs for cytokines and chemokines in lungs. Lung homogenates were prepared at the indicated times after infection with 5×10^4 CFU of strain NMU112. At each time point, samples from three mice in each group were used. (A) KC and MCP-1 protein levels in samples from individual mice were measured in triplicate by ELISA. Data are representative of results from two separate experiments, and the error bars represent SEM. *, P of <0.05; **, P of <0.025; ***, P of <0.01 for comparison with controls. (B) Total RNA was extracted from the left lung at 8 and 24 h after infection, and 1 μg of total RNA was transcribed into cDNA. Quantitative real-time PCR analysis was performed with the TaqMan universal PCR master mix by using 1 μl of cDNA in a 20-μl final reaction mixture volume. The levels of target cytokine and chemokine messages were expressed as ratios of the target mRNA to GAPDH mRNA. Data are representative of results from three separate experiments, and the error bars represent SEM. *, $P < 0.05$. Between the RXM-treated and the control groups, the message for MCP-1 was significantly higher ($P < 0.05$) in the treated group, while that for KC was significantly higher ($P < 0.05$) in the control group. (C) Syndecan-1 levels in BALF were measured by ELISA. At indicated time points, BALF was harvested from three mice in each group, and individual samples were assayed in triplicate. Data are representative of results from three separate experiments, and the error bars represent SEM. *, $P < 0.01$; **, $P < 0.005$.

assessed by zymography was significantly reduced in the lungs of RXM-treated mice during the first 48 h; therefore, RXM is thought to reduce syndecan-1 shedding by suppressing MMP-7 expression. MMP-7 is also involved in reepithelialization by repairing injured tracheas (9), but RXM did not affect the process of repairing injured lung tissue as judged from histological examinations showing that inflamed pulmonary tissue in the treated mice returned to normal at 3 days postinfection in this study.

CXC chemokines are essential for PMN recruitment to the lungs following inflammatory injury in mice. KC (a homologue of human interleukin-8) has been shown to bind to syndecan-1 ectodomains; an MMP-7-mediated shedding of syndecan-1–KC complexes from the mucosal surface directs and confines PMN influx to sites of injury in the lungs (30). RXM reduced the expression of KC and MMP-7, which resulted in lower concentrations of both proteins in BALF from RXM-treated mice than in that from the controls. In contrast, MCP-1 message and protein levels increased in the lungs of RXM-treated mice after infection. Differences in the expression levels of mRNAs for KC and MCP-1 in the lungs of the treated mice may be due to the ability of 14-membered-ring macrolides to selectively suppress the production of cytokines at transcriptional levels (1, 2, 26, 44). Despite the reduced levels of PMN accumulation, RXM enhanced local resistance to pneumococci, even though the RXM concentrations in the lungs and blood were lower than the MIC. The degrees of PMN infiltration during the first 8 h and the magnitudes of the bacterial burdens in the two groups were similar until 24 h after infection, while numbers of CFU in the treated mice became significantly lower than those in the controls after 48 h of infection. Enhanced MCP-1 production may account for the

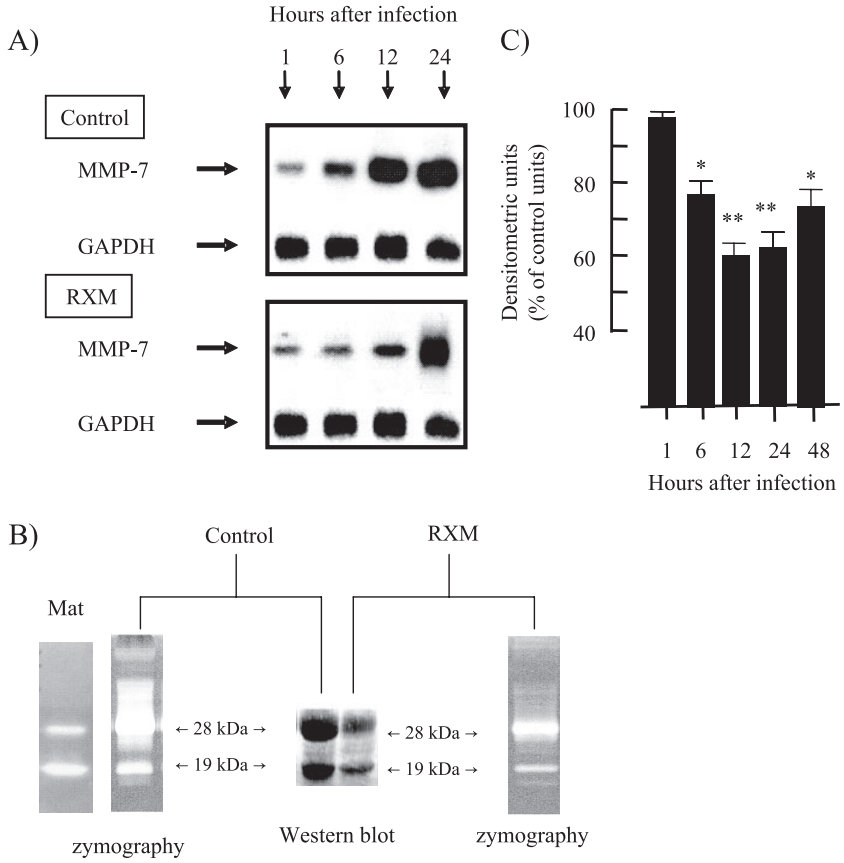

FIG. 7. Effect of RXM on the expression and activation of MMP-7 in the lung. (A) Expression of MMP-7 mRNA in the lungs after infection. cDNA was generated by one-step RT-PCR using total RNA and specific primers for mouse MMP-7 and GAPDH genes, and PCR products were evaluated by Southern hybridization using cDNA probes specific for MMP-7 and GAPDH genes. At each time point, RNA was extracted from three mice and individual RNA samples were assayed. The same experiments were performed twice. Representative results are shown. (B) Casein zymography using NOVEX 4 to 16% zymogram blue casein gels was performed with the extract (100 μg) of lungs from mice at 12 h postinfection; activated recombinant human MMP-7 (Mat; R & D Systems) was included as a positive control. A precursor (28 kDa) and a mature form (19 kDa) were identified by Western blotting using anti-MMP-7 polyclonal antiserum. Extracts of lungs from three mice were prepared, and individual samples were assayed. Representative results are shown. (C) Casein zymography was performed at the indicated time points after infection. The zymograms were inverted and scanned, and caseinolytic activity was determined by scanning the lysis band in the area corresponding to a mature form (19 kDa) of MMP-7 by using a computerized densitometer. Data are representative of results from three separate experiments and are expressed as mean percentages of the activity in the lungs of infected control mice. The error bars represent SEM (*n*, 5 to 6 at each time point). *, *P* of <0.05; **, *P* of <0.025 for comparison with the activity levels in the extracts from infected control mice at the indicated time points.

increase in the infiltration of mononuclear cells after 12 h; this increase likely contributed to the reduction in bacterial burdens in the lungs of the treated mice after 48 h of infection, since mononuclear cells exhibit enhanced phagocytic bactericidal activity against type 3 pneumococci by adhering to the extracellular matrix (37). In contrast, enhanced accumulation of PMN at the confined area is thought to damage lung tissue by releasing proteases and reactive oxygen species (6, 46), which might explain the markedly damaged lung tissue of the control mice in this study. Treatment with RXM apparently reduced PMN recruitment in the lungs, which was likely to allow inflammatory tissue to return to normal after day 3 of infection. Actually, Kawashima et al. (24) have shown that a 14-membered-ring macrolide inhibits bleomycin-induced acute lung injury through its suppressive activity toward PMN recruitment. In addition, 14-membered-ring macrolides regulate the ability of leukocytes to produce proteases and reactive oxygen species (29), both of which are thought to induce tissue damage. Since there has been abundant evidence to show the direct effect of macrolides on neutrophils and macrophages (reviewed in references 8 and 29), the modulation of inflammatory processes by RXM observed in this study may also include the direct effects of this macrolide on the function of these phagocytes. The enhancement of local resistance, with the delayed development of bacteremia, is therefore thought to be due to the combined effects of RXM on the phagocytes and MMP-7.

The concentrations of RXM in the plasma and lung tissue in mice receiving a daily dose of 5 mg/kg were much lower than the MIC at the time point of infection and in subsequent days of infection. Such low concentrations of RXM are unable to decrease the synthesis of pneumococcal virulence factors, which is consistent with the findings in a report by other investigators (13). In connection with this information, we chose 5 μg/ml as the drug concentration for the in vitro assay of PLY and PspA suppression (Fig. 1). This concentration of the mac-

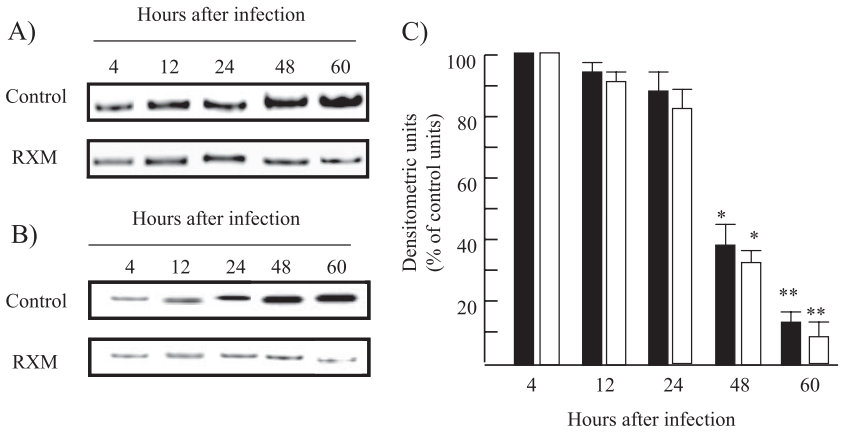

FIG. 8. Effect of RXM on the production of PLY and PspA by pneumococci in the lung. Western blot analysis for PLY (A) and PspA (B) in lung extracts from RXM-treated mice and controls was performed at the indicated time points after infection. Extracts of lungs from three mice were prepared, and samples from individual mice were assayed. Representative results are shown. (C) The signal intensity of each band for PLY (black bars) and PspA (white bars) was determined using a computerized densitometer. Data are representative of three separate experiments and are expressed as mean percentages of the intensity of each band in the lung extracts from infected control mice. The error bars represent SEM (n, 5 to 6 at each time point). *, P of <0.025; **, P of <0.01 for comparison with controls.

rolides tested is higher than their levels in plasma in mice and possibly higher than clinically achievable concentrations. However, macrolide concentrations are reported to be routinely higher in pulmonary epithelial lining fluid and phagocytes than in plasma (40). Thus, we performed the in vitro assay of PLY and PspA suppression (Fig. 1) at drug concentrations higher than the plasma drug levels in mice. Furthermore, we think that the results obtained from the suppression assay for both proteins might be relevant to the effect of RXM in the lungs, since macrolide-resistant *S. pneumoniae* must first encounter epithelial lining fluid and phagocytes in the airway. Although neither PLY nor PspA levels in the lungs were reduced during the first 24 h of infection, the levels of these proteins were significantly decreased at 48 and 60 h postinfection. This reduction seemed to be due to the decrease in numbers of CFU after 48 h of infection.

As already mentioned, a marked PMN-dominant response upon infection with macrolide-resistant *S. pneumoniae* may cause severe tissue damage, which may possibly affect local resistance and also retard recovery from tissue damage. The inhibition of neutrophil functions (e.g., chemotaxis and oxidative burst) and the stimulation of macrophage functions (e.g., phagocytosis of encapsulated pneumococci as well as apoptotic cells) may contribute to the local resistance conferred by RXM in addition to the down-regulation of MMP-7. Furthermore, the decrease in numbers of CFU in the lungs occurred at least 24 h after the appearance of anti-inflammatory effects (such as changes in levels of MMP-7, MPO activity, and infiltrating cells). Therefore, the regulation of the acute inflammatory response during the initial phase of infection with macrolide-resistant *S. pneumoniae* must be a prerequisite to the observed enhancement of local resistance by sub-MIC levels of RXM. Furthermore, such regulatory activity of RXM might be expressed independently of an effect on pneumococcal virulence factors, since this macrolide apparently reduced inflammatory responses against killed pneumococci.

Pneumococcal invasion into the bloodstream occurred in control mice within the first 12 h. RXM retarded this invasion,

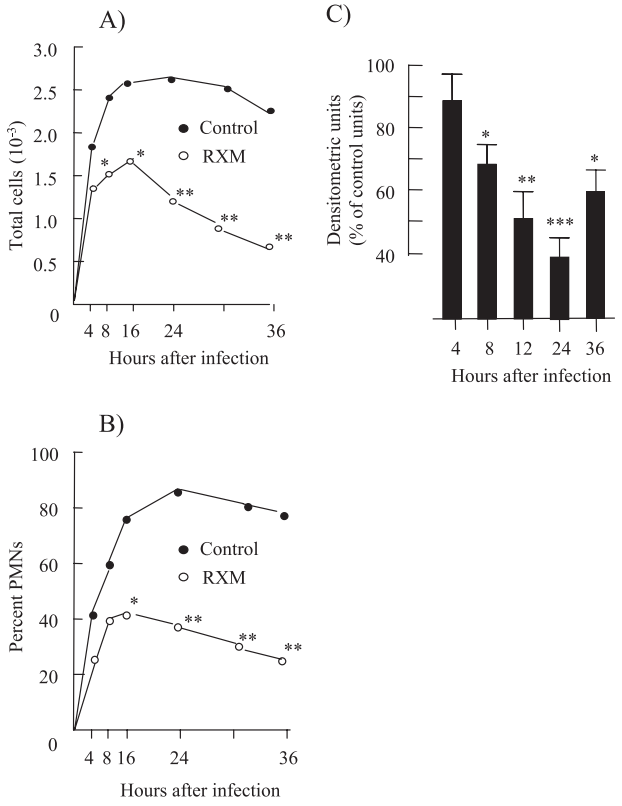

FIG. 9. Effect of RXM on local responses in the airway after inoculation with UV ray-killed NMU112. BALF was harvested from the RXM-treated and control mice after inoculation with 10^8 UV ray-killed pneumococci. (A) Numbers of infiltrating cells in BALF. (B) The percentages of PMNs among the infiltrating cells at the indicated times were determined (n, 5 per group at each time point). Data are presented as the mean value from two separate experiments. *, P of <0.05; **, P of <0.01 for comparison with controls. (C) Levels of MMP-7 activity in lung extracts were assessed by casein zymography, and comparative activity was determined by scanning the lysis band in the area corresponding to a 19-kDa form by using a computerized densitometer (as described in the legend to Fig. 7). Data obtained from two separate experiments (n, 4 per group at each time point) are presented as the means ± SEM. *, P of <0.05; **, P of <0.025; ***, P of <0.01 for comparison with controls.

although the numbers of CFU in the lungs of the treated mice remained at the same levels as those in the lungs of the controls during this period of time. For pneumococcal invasion into the bloodstream, the integrity of the extracellular matrix of the basement membrane on blood vessels must be degraded; this process may require both bacterial and host factors in lung tissue (4). In RXM-treated mice, bacteremia occurred after day 3 of infection, despite the decreased numbers of CFU in the lung. There are two important points to note that have already been mentioned: sub-MIC levels of RXM (i) suppressed inflammatory responses to killed pneumococci in the lungs and (ii) did not reduce pneumococcal virulence factors in the lungs. Considering these points together, it is plausible that the subtherapeutic dose of RXM can delay the bloodstream invasion by macrolide-resistant *S. pneumoniae* through the regulatory effects of RXM on host factors including MMP-7 and phagocytes, although the present study did not prove that the down-regulation of MMP-7 by RXM directly contributes to the enhancement of mouse resistance. In addition, we speculate that concentrations of RXM in tissue after day 3 of infection may be lower than those able to regulate local responses in the lungs.

Finally, we found that subtherapeutic doses of ERY, CLR, and AZM also increased mouse survival times. At low doses, treatment with these macrolides achieved only the same MST as 5 mg/kg/day of RXM, despite their ability to affect pneumococcal virulence factors. This result might be accounted for by the assumption that the low dosages of ERY, CLR, and AZM used would not yield concentrations in the epithelial lining fluid and/or phagocytes high enough to suppress PLY and PspA production. At higher dosages, however, these macrolides increased MSTs by only 1.49 to 1.63 days compared to the MST corresponding to the low dose of each antibiotic; this increase, though not significant, may be due to their additional effect on pneumococcal virulence factors. These data are consistent with the findings in the report by Fukuda et al. (12) showing that both CLR and AZM increase the survival times of immunocompetent mice after infection with a high level of macrolide-resistant *S. pneumoniae*. Nevertheless, none of the macrolides tested in this study were able to protect mice from bloodstream invasion by macrolide-resistant *S. pneumoniae*, which resulted in the death of all infected mice. In neutropenic mice, however, the efficacy of CLR and AZM against clinical isolates of macrolide-resistant *S. pneumoniae* varied with the level of macrolide resistance of the pathogen (20), which indicates that the enhancement of local resistance against macrolide-resistant *S. pneumoniae* by sub-MIC levels of macrolide compounds may require an immunocompetent status of the hosts. Furthermore, this fact strongly suggests that the direct antimicrobial activity of macrolides should be necessary for complete protection against a high level of macrolide-resistant *S. pneumoniae*, regardless of the degree of innate immunity in the hosts.

In conclusion, we have demonstrated that subtherapeutic doses of RXM favorably modify the initial response to macrolide-resistant *S. pneumoniae* in the airways of immunocompetent mice, thereby retarding the development of pneumococcal bacteremia. In addition, this modification appears to be associated with the regulatory effects of RXM on MMP-7 expression and activation and phagocytic cell functions and can be achieved without affecting pneumococcal virulence factors.

ACKNOWLEDGMENTS

We are grateful to Yasunori Tanaka (Bioengineering Japan, Tokyo) for technical assistance with the preparation of mouse MMP-7 probes. We thank Hiroshi Yoshimoto and Kinuko Nakagawa for help with the determination of the roxithromycin concentrations by HPLC.

This study was supported by a grant-in-aid for scientific research from the Japanese Society for the Promotion of Science (grant 15659239).

REFERENCES

1. **Abe, S., H. Nakamura, S. Inoue, H. Saito, S. Kato, N. Mukaida, K. Matsushima, and H. Tomoike.** 2000. Interleukin-8 gene repression by clarithromycin is mediated by the activator protein-1 binding site in human bronchial epithelial cells. Am. J. Respir. Cell Mol. Biol. **22:**51–60.
2. **Abeyama, K., K. Kawahara, S. Iino, T. Hamada, S. Arimura, K. Matsushita, T. Nakajima, and I. Maruyama.** 2003. Antibiotic cyclic AMP signaling by "primed" leukocytes confers anti-inflammatory cytoprotection. J. Leukoc. Biol. **74:**908–915.
3. **Alexander, C. M., F. Reichsman, M. T. Hinkes, J. Lincecum, K. A. Becker, S. Cumberledge, and M. Bernfield.** 2000. Syndecan-1 is required for Wnt-1-induced mammary tumorigenesis in mice. Nat. Genet. **25:**329–332.
4. **Allen, B. L., B. Katz, and M. Hook.** 2002. *Streptococcus anginosus* adheres to vascular endothelium basement membrane and purified extracellular matrix proteins. Microb. Pathog. **32:**191–204.
5. **Anzueto, A., M. S. Niederman, G. S. Tillotson, and the Bronchitis Study Group.** 1998. Etiology, susceptibility, and treatment of acute bacterial exacerbations of complicated chronic bronchitis in the primary care setting: ciprofloxacin 750 mg b.i.d. versus clarithromycin 500 mg b.i.d. Clin. Ther. **20:**885–900.
6. **Auten, R. L., M. H. Whorton, and N. S. Mason.** 2002. Blocking neutrophil influx reduces DNA damage in hyperoxia-exposed newborn rat lung. Am. J. Respir. Cell Mol. Biol. **26:**391–397.
7. **Bui, K. Q., M. A. Banevicius, C. H. Nightingale, R. Quintiliani, and D. P. Nicolau.** 2000. *In vitro* and *in vivo* influence of adjunct clarithromycin on the treatment of mucoid *Pseudomonas aeruginosa*. J. Antimicrob. Chemother. **45:**57–62.
8. **Culić, O., V. Erakovic, and M. J. Parnham.** 2001. Anti-inflammatory effects of macrolide antibiotics. Eur. J. Pharmacol. **429:**209–229.
9. **Dunsmore, S. E., U. K. Saarialho-Kere, J. D. Roby, C. L. Wilson, L. M. Matrisian, H. G. Welgus, and W. C. Parks.** 1998. Matrilysin function and expression in airway epithelium. J. Clin. Investig. **102:**1321–1331.
10. **Edwards, D., C. Parfett, and D. Denhardt.** 1985. Transcriptional regulation of two serum-induced RNAs in mouse fibroblasts. Mol. Cell. Biol. **5:**3280–3288.
11. **Finch, R.** 2001. Community-acquired pneumonia: the evolving challenge. Clin. Microbiol. Infect. **7**(Suppl. 3)**:**30–38.
12. **Fukuda, Y., K. Yanagihara, Y. Higashiyama, Y. Miyazaki, Y. Hirakata, H. Mukae, K. Tomono, Y. Mizuta, K. Tsukamoto, and S. Kohno.** 2006. Effects of macrolides against pneumolysin of macrolide-resistant *Streptococcus pneumoniae*. Eur. Respir. J. **27:**1020–1025.
13. **Gemmell, C. G., and M. McLeod.** 1992. The effect of roxithromycin on the virulence of gram-positive cocci. Diagn. Microbiol. Infect. Dis. **15**(Suppl. 1)**:**67S–70S.
14. **Gibbs, D. F., R. L. Warner, S. J. Weiss, K. J. Johnson, and J. Varani.** 1999. Characterization of matrix metalloproteinases produced by rat alveolar macrophages. Am. J. Respir. Cell Mol. Biol. **20:**1136–1144.
15. **Hamada, K., E. Kita, M. Sawaki, K. Mikasa, and N. Narita.** 1995. Antitumor effect of erythromycin in mice. Chemotherapy **41:**59–69.
16. **Hamada, K., K. Mikasa, Y. Yunou, T. Kurioka, T. Majima, N. Narita, and K. Kita.** 2000. Adjuvant effect of clarithromycin on chemotherapy for murine lung cancer. Chemotherapy **46:**49–61.
17. **Haro, H., H. C. Crawford, B. Fingleton, J. R. MacDougall, K. Shinomiya, D. M. Spengler, and L. M. Matrisian.** 2000. Matrisian, matrix metalloproteinase-3-dependent generation of a macrophage chemoattractant in a model of herniated disc resorption. J. Clin. Investig. **105:**133–141.
18. **Hashimoto, N., T. Kawabe, T. Hara, T. Imaizumi, H. Wakayama, H. Saito, K. Shimokata, and Y. Hasegawa.** 2001. Effect of erythromycin on matrix metalloproteinase-9 and cell migration. J. Lab. Clin. Med. **137:**176–183.
19. **Hirakata, Y., M. Kaku, R. Mizukane, K. Ishida, N. Furuya, T. Matsumoto, K. Takeda, and K. Yamaguchi.** 1992. Potential effects of erythromycin on host defense systems and virulence of *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **36:**1922–1927.
20. **Hoffman, H. L., M. E. Klepser, E. J. Ernst, C. R. Petzold, L. M. Sa'adah, and G. V. Doern.** 2003. Influence of macrolide susceptibility on efficacies of clarithromycin and azithromycin against *Streptococcus pneumoniae* in a murine lung infection model. Antimicrob. Agents Chemother. **47:**739–746.

21. **Kanai, K., K. Asano, T. Hisamitsu, and H. Suzaki.** 2004. Suppression of matrix metalloproteinase production from nasal fibroblasts by macrolide antibiotics *in vitro*. Eur. Respir. J. **23:**671–678.
22. **Kasahara, K., E. Kita, K. Maeda, K. Uno, K. Takahashi, M. Konishi, E. Yoshimoto, K. Murakawa, E. Kita, and H. Kimura.** 2005. Macrolide resistance of *Streptococcus pneumoniae* isolated during long-term macrolide therapy: difference between erythromycin and clarithromycin. J. Infect. Chemother. **11:**112–114.
23. **Kasahara, K., K. Maeda, K. Mikasa, K. Uno, K. Takahashi, M. Konishi, E. Yoshimoto, K. Murakawa, E. Kita, and H. Kimura.** 2005. Clonal dissemination of macrolide-resistant and penicillin-susceptible serotype 3 and penicillin-resistant Taiwan 19F-14 and 23F-15 *Streptococcus pneumoniae* isolates in Japan: a pilot surveillance study. J. Clin. Microbiol. **43:**1640–1645.
24. **Kawashima, M., J. Yatsunami, Y. Fukuno, M. Nagata, M. Tominaga, and S. Hayashi.** 2002. Inhibitory effects of 14-membered ring macrolide antibiotics on bleomycin-induced acute lung injury. Lung **180:**73–89.
25. **Kita, E., K. Mikasa, and K. Kasahara.** 2003. Syndecan shedding from epithelial cells affects host defense against respiratory infection. Int. Congr. Ser. **1257:**21–26.
26. **Kobayashi, M., T. Shimauchi, R. Hino, and Y. Tokura.** 2004. Roxithromycin downmodulates Th2 chemokine production by keratinocytes and chemokine receptor expression on Th2 cells: its dual inhibitory effects on the ligands and the receptors. Cell. Immunol. **228:**27–33.
27. **Kobayashi, T., K. Tateda, T. Matsumoto, S. Miyazaki, A. Watanabe, T. Nukiwa, and K. Yamaguchi.** 2002. Macrolide-treated *Pseudomonas aeruginosa* induces paradoxical host responses in the lungs of mice and a high mortality rate. J. Antimicrob. Chemother. **50:**59–66.
28. **Konno, S., K. Asano, M. Kurokawa, K. Ikeda, K. Okamota, and M. Adachi.** 1994. Antiasthmatic activity of a macrolide antibiotic, roxithromycin: analysis of possible mechanisms *in vitro* and *in vivo*. Int. Arch. Allergy Immunol. **105:**308–316.
29. **Labro, M. T.** 2004. Cellular and molecular effects of macrolides on leukocyte function. Curr. Pharm. Des. **10:**3067–3080.
30. **Li, Q., P. W. Park, C. L. Wilson, and W. C. Parks.** 2002. Matrilysin shedding of syndecan-1 regulates chemokine mobilization and transepithelial efflux of neutrophils in acute lung injury. Cell **111:**635–646.
31. **Lonks, J. R., J. Garau, L. Gomez, M. Xercavins, A. O. de Echaguen, I. F. Gareen, P. T. Reiss, and A. A. Medeiros.** 2002. Failure of macrolide antibiotic treatment in patients with bacteremia due to erythromycin-resistant *Streptococcus pneumoniae*. Clin. Infect. Dis. **35:**556–564.
32. **Lonks, J. R., J. Garau, and A. A. Medeiros.** 2002. Implications of antimicrobial resistance in the empirical treatment of community-acquired respiratory tract infections: the case of macrolides. J. Antimicrob. Chemother. **50**(Suppl. S2)**:**87–92.
33. **Lopez-Boado, Y. S., C. L. Wilson, L. V. Hooper, J. I. Gordon, S. J. Hultgren, and W. C. Parks.** 2000. Bacterial exposure induces and activates matrilysin in mucosal epithelial cells. J. Cell Biol. **148:**1305–1315.
34. **Mandell, L. A., J. G. Bartlett, S. F. Dowell, T. M. File, Jr., D. M. Musher, and C. Whitney.** 2003. Update of practice guidelines for the management of community-acquired pneumonia in immunocompetent adults. Clin. Infect. Dis. **37:**1405–1433.
35. **Mizukane, R., Y. Hirakata, M. Kaku, Y. Ishii, N. Furuya, K. Ishida, H. Koga, S. Kohno, and K. Yamaguchi.** 1994. Comparative *in vitro* exoenzyme-suppressing activities of azithromycin and other macrolide antibiotics against *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **38:**528–533.
36. **National Committee for Clinical Laboratory Standards.** 1993. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. Approved standard M7–A5, 5th ed. National Committee for Clinical Laboratory Standards, Villanova, Pa.
37. **Newman, S. L., and M. A. Tucci.** 1990. Regulation of human monocyte/macrophage function by extracellular matrix. Adherence of monocytes to collagen matrices enhances phagocytosis of opsonized bacteria by activation of complement receptors and enhancement of Fc receptor function. J. Clin. Investig. **86:**703–714.
38. **Park, P. W., G. B. Pier, M. T. Hinkes, and M. Bernfield.** 2001. Exploitation of syndecan-1 shedding by *Pseudomonas aeruginosa* enhances virulence. Nature **411:**98–102.
39. **Patel, K. B., D. Xuan, P. R. Tessier, J. H. Russomanno, R. Quintiliani, and C. H. Nightingale.** 1996. Comparison of bronchopulmonary pharmacokinetics of clarithromycin and azithromycin. Antimicrob. Agents Chemother. **40:**2375–2379.
40. **Rodvold, K. A., M. H. Gotfried, L. H. Danziger, and R. J. Servi.** 1997. Intrapulmonary steady-state concentrations of clarithromycin and azithromycin in healthy adult volunteers. Antimicrob. Agents Chemother. **41:**1399–1402.
41. **Rostand, K. S., and J. D. Esko.** 1997. Microbial adherence to and invasion through proteoglycans. Infect. Immun. **65:**1–8.
42. **Sakata, K., H. Yajima, K. Tanaka, Y. Sakamoto, K. Yamamoto, A. Yoshida, and Y. Dohi.** 1993. Erythromycin inhibits the production of elastase by *Pseudomonas aeruginosa* without affecting its proliferation *in vitro*. Am. Rev. Respir. Dis. **148:**1061–1065.
43. **Suzaki, H., K. Asano, S. Ohki, K. Nanai, T. Mizutani, and T. Hisamitsu.** 1999. Suppressive activity of a macrolide antibiotic, roxithromycin, on proinflammatory cytokine production *in vitro* and *in vivo*. Mediators Inflamm. **8:**199–204.
44. **Takizawa, H., M. Desaki, T. Ohtoshi, S. Kawasaki, T. Kohyama, M. Sato, M. Tanaka, T. Kasama, K. Kobayashi, J. Nakajima, and K. Ito.** 1997. Erythromycin modulates IL-8 expression in normal and inflamed human bronchial epithelial cells. Am. J. Respir. Crit. Care Med. **156:**266–271.
45. **Tateda, K., Y. Ishii, T. Matsumoto, T. Kobayashi, S. Miyazaki, and K. Yamaguchi.** 2000. Potential of macrolide antibiotics to inhibit protein synthesis of *Pseudomonas aeruginosa*: suppression of virulence factors and stress response. J. Infect. Chemother. **6:**1–7.
46. **Thatcher, T. H., N. A. McHugh, R. W. Egan, R. W. Chapman, J. A. Hey, C. K. Turner, M. R. Redonnet, K. E. Seweryniak, P. J. Sime, and R. P. Phipps.** 2005. Role of CXCR2 in cigarette smoke-induced lung inflammation. Am. J. Physiol. Lung Cell. Mol. Physiol. **289:**L322–L328.
47. **Wilson, C. L., K. J. Heppner, L. A. Rudolph, and L. M. Matrisian.** 1995. The metalloproteinase matrilysin is preferentially expressed by epithelial cells in a tissue-restricted pattern in the mouse. Mol. Biol. Cell **6:**851–869.